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# Hepatocyte Growth Factor (HGF) Receptor Expression and Role of HGF during Embryonic Mouse Testis Development

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**The hepatocyte growth factor (HGF) receptor, c-met, transduces the HGF multiple biological activities. During embryonic development the system HGF/c-met regulates the morphogenesis of different organs and tissues. In this study we examined c-met gene expression during mouse testis development and, by means of Northern blot and *in situ* hybridization, we report the receptor expression pattern. C-met expression is not detectable in male genital ridges isolated from embryos at 11.5 days postcoitum (dpc). In testes isolated from 12.5 and 13.5 dpc, c-met expression is detectable and essentially localized in the developing cords. Male genital ducts do not express c-met at the reported ages, whereas female ducts appear c-met positive. Moreover, we report that HGF is able to induce testicular morphogenesis *in vitro*. Male genital ridges isolated from embryos at 11.5 dpc are morphologically nonorganized. Culturing 11.5 dpc urogenital ridges in the presence of HGF we obtained testis organization and testicular cord formation. Our data demonstrate that c-met is expressed during the beginning period of testis differentiation and that HGF is able to support testicular differentiation *in vitro*. All these data indicate that this growth factor, besides its role as mitogenic factor, plays a fundamental role during testicular cord formation probably inducing cell migration and/or cell differentiation.** 

***Key Words:*** **HGF/SF; c-MET; mouse testis; testis development.**

## INTRODUCTION

Hepatocyte growth factor (HGF) is a pleiotropic cytokine originally purified as a potent mitogen for hepatocytes (Nakamura *et al.*, 1984, 1989) and subsequently identified as a scatter factor (Stoker *et al.*, 1987; Weidner *et al.*, 1993a), exerting multiple biological activities on various epithelial cells (reviewed by Matsumoto and Nakamura, 1993, 1996; Zarnegar and Michalopoulos, 1995; Trusolino *et al.*, 1998). The receptor for HGF (c-met, HGFR), encoded by the c-met protooncogene, is a 190-kDa glycoprotein with tyrosine kinase activity, expressed in a variety of normal and malignant mammalian tissues (Park *et al.*, 1987; Iyer *et al.*, 1990; Di Renzo *et al.*, 1991). C-met specifically binds to HGF transducing its multiple biological activities which includes cell motility and proliferation (Weidner *et al.*, 1993b; Hartmann *et al.*, 1994). C-met also regulates embryonic morphogenesis and mediates signal exchange between mesenchyme and epithelia during mouse development (Sonnenberg *et al.*, 1993). Mesenchymal to epithelial conversion has been induced as a consequence of HGF overexpression (Tsarfaty *et al.*, 1994) and epithelial conversion of mesenchymal cells isolated from metanephros has been obtained after stimulation with HGF (Karp *et al.*, 1994). Recently the role of HGF as mediator of epithelial–mesenchymal interaction has been underlined by a paper demonstrating that HGF is expressed in the mesenchyme and c-met is expressed in the epithelium of the developing lung (Ohmichi *et al.*, 1998). In the same paper the morphogenetic role of HGF is also demonstrated since, in organ culture, exogenously added HGF is able to stimulate branching morphogenesis of the fetal lung, whereas other growth factors, presumably involved in fetal lung development, do not exert the same morphogenetic effect. The morphogenic effect of HGF on epithelial cells, resulting in the formation of tubules and gland-like structures, has been previously described also in cells derived from kidney and from mammary gland (Montesano *et al.*, 1991; Soriano *et al.*, 1995; Brinkmann *et al.*, 1995). The important role of the HGF and HGF receptor in the morphogenesis of epithelial organs during mouse development has been established looking at the HGF- or c-met-null mutant embryos, in which the liver is reduced in size and the placenta is abnormal, unable to nourish the embryos in the second part of gestation

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(Schmidt *et al.*, 1995; Uehara *et al.*, 1995; as a review, see Birchmeier and Gherardi, 1998).

The molecular mechanisms throughout c-met which induce its biological effects are not completely clarified although it is known that motility response requires PI 3-kinase (Royal and Park, 1995) and Ras-Rac/Rho pathways activation (Hartmann *et al.*, 1994; Sachs *et al.*, 1996; Ridley *et al.*, 1995) whereas the growth response requires the activation of the Ras-mitogen-activated proteins (Ponzetto *et al.*, 1996). The morphogenetic response has been obtained when the "signal transducers and activator of transcription" (STAT) pathway was activated, as demonstrated in canine kidney epithelial (MDCK) cells in which STAT3 is phosphorylated and translocated to the nucleus as a consequence of HGF stimulation (Boccaccio *et al.*, 1998).

The role of HGF in the genital ridge development has not been studied and, at the moment, it is unknown if HGFR is expressed during the embryonic testis development. In the adult mouse testis HGFR expression was not detected (Iyer *et al.*, 1990), whereas it was detected in the adult human and prepuberal rat testis (Depuydt *et al.*, 1996; Catizone *et al.*, 1999). In the present paper, c-met expression during testis morphogenesis has been studied and we report that, in the embryonic mouse testis, c-met is expressed and it is functionally active, since HGF is able to sustain "*in vitro*" testis morphogenesis.

## MATERIALS AND METHODS

### *Animals*

CD-1 mice embryos were used for the experiments. For determination of the age of the embryos, the morning after vaginal plug formation was considered as day 0.5 of embryonic development. On day 11.5 of pregnancy, male and female gonads are morphologically indistinguishable and the embryos were sexed scoring the presence of the Barr bodies according to Palmer and Burgoyne (1991).

### *RNA Isolation and Northern Blot Analysis*

Various tissues from embryonic CD-1 mice were dissected and RNA was extracted according to Chomczynski and Sacchi (1987). The integrity of the RNA was tested through the presence of the ribosomal species in formaldehyde denaturing gels. Northern blot analysis using 30 $\mu$g of RNA in each lane was performed on 1% agarose/formaldehyde gels and transferred to Hybond-N+ membrane (Amersham-Italia, Milan, Italy). Prehybridization, hybridization, and washings were performed according to the conditions suggested by the supplier. The membrane was exposed on X-ray film. Mouse met cDNA (kindly provided by Dr. C. Ponzetto, Torino University) was labeled using a random primer labeling kit (Gibco BRL, Life Technologies, Gaithersburg, MD). Relative differences in hybridization were determined by scanning densitometry of autoradiograms. C-met expression in total RNA was normalized to the signal for the constitutively expressed glyceraldeyde-3-phosphate dehydrogenase (GAPDH).

### *Whole Mount in Situ Hybridization*

*In situ* hybridization (ISH) was performed on embryonic testes fixed by overnight immersion in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, at 4°C and washed twice in PBS for 1 h. The samples were treated with 10 $\mu$g/ml proteinase K in PBS containing 0.1% Tween for 10–20 minutes at room temperature. Antisense and sense riboprobes were generated to the mouse c-met c-DNA (kindly provided by Dr. C. Ponzetto, Torino University), subcloned in Bluescript II Sk, by *in vitro* transcription by T3 and T7 RNA polymerase in the presence of digoxigenin-labeled UTP following the manufacturer's instructions. Probes were diluted in hybridization mix at 1 $\mu$g/ml and 1 ml of hybridization mix was applied to the samples. Detection of c-met mRNA by *in situ* hybridization to whole mounts was carried out according to Wilkinson and Nieto (1993). The hybrids bound to alkaline phosphatase conjugated anti-digoxigenin antibody were visualized by a color reaction mixture containing 1% Tween 20, 2 mM Levamisole in BM purple AP substrate (Boehringer, Mannheim, Germany) and color was allowed to develop for 3–5 h in the dark. The reaction was stopped by incubation with PBS, 0.1% Tween 20, 10 mM ethylenediaminetetraacetic acid (EDTA) for 10 min. Samples were fixed with 4% formaldehyde in PBS overnight at 4°C and stored in PBS containing 0.1% sodium azide at 4°C. For sectioning, the samples were maintained in PBS, 7% sucrose for 5–10 min at 4°C and then in PBS, 15% sucrose for 5–10 min at 4°C; successively the samples were maintained in PBS, 15% sucrose, 7% gelatin at 37°C to achieve the complete embedding of the samples. The samples were then included in the latter solution which is solid at room temperature and frozen in liquid nitrogen. The samples were sectioned, viewed, and photographed by a light microscope (Zeiss Axioplan).

### *Organ Culture*

The urogenital ridges (UGR) were isolated from 11.5 days post-coitum (dpc) male embryos and cultured for 4 days on steel grids previously coated with 2% agar. Grids were then placed in organ culture dishes (Falcon) with 0.8 ml of medium necessary to wet the grid. The chemically defined medium utilized was Dulbecco's modified Eagle's medium (Gibco, DMEM) supplemented with glutamine (2 mM), Hepes (15 mM), nonessential amino acids, penicillin (100 IU/ml), and streptomycin (100 $\mu$g/ml). Ten percent fetal calf serum (FCS, Gibco), HGF (200–400 U/ml of pure recombinant HGF kindly provided by Dr. C. Ponzetto, Torino University), transforming growth factor $\beta$ (TGF$\beta$, Sigma Chemical Co., 5 ng/ml), basic fibroblast growth factor (bFGF, Boehringer, 10 ng/ml), platelet-derived growth factor (PDGF-BB, Sigma, 100 ng/ml), and follicle-stimulating hormone (FSH, NIH ov-FSH S-17, 100 ng/ml) were added to the culture medium when indicated. UGR were cultured at 37°C in a humidified atmosphere of 5% $CO_2$ in air. After culture, tissues were fixed overnight in 4% paraformaldehyde in PBS, pH 7.4, at 4°C and washed twice in PBS for 1 h. After dehydration, samples were treated with xylene, embedded in paraffin, and sectioned at a thickness of 5 $\mu$m. The sections were dewaxed, stained with carmalum, viewed, and photographed by a light microscope (Zeiss Axioplan).

### *Bromodeoxyuridine Labeling of Organ Cultures*

Testes were isolated from 13.5 dpc embryos and cultured as above indicated in the presence of medium alone, 10% serum, or HGF (200 U/ml) for 16 h. Bromodeoxyuridine (BrdU) was then added to the culture medium for 5 h before harvesting. The protocol specified by the manufacturer (cell proliferation kit, Amersham Corp.) was used. Samples were washed twice in PBS, fixed overnight in Bouin's fixative, dehydrated, embedded in paraffin, sectioned, and processed according to the manufacturer to detect BrdU-labeled cells.

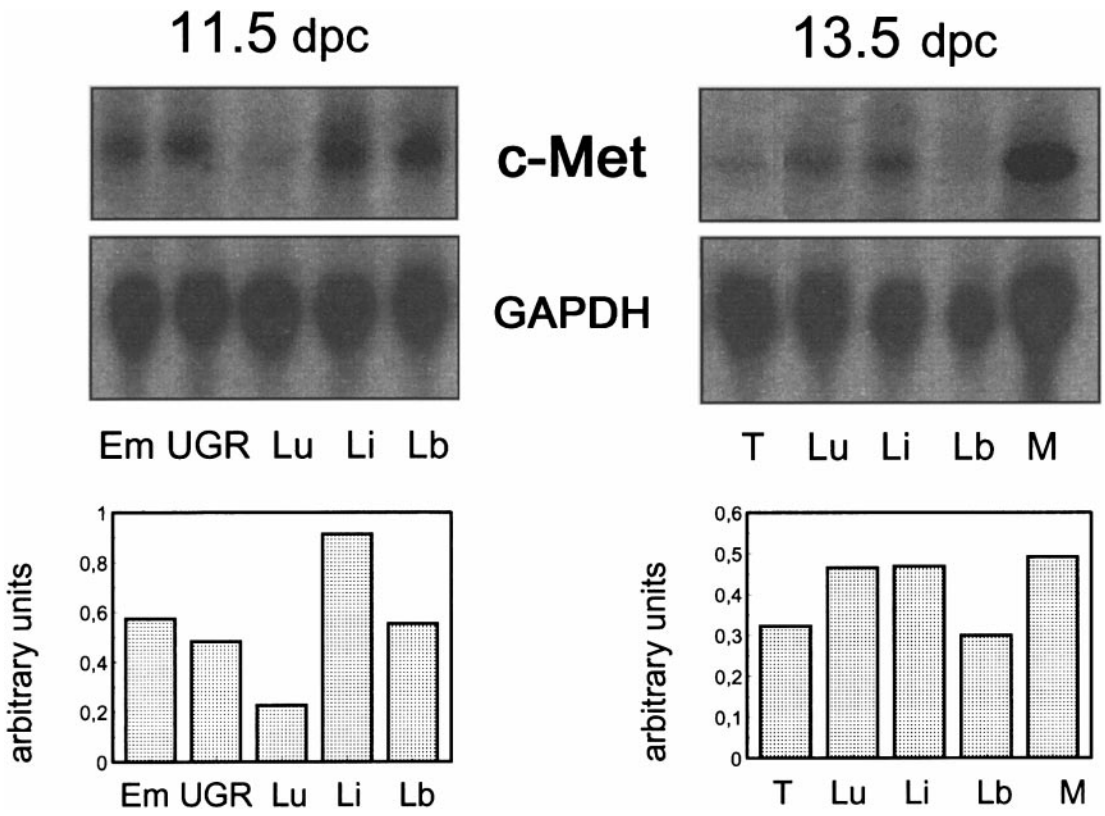


**FIG. 1.** Expression of c-met mRNA in the developing male gonad. (Top) Northern blot analysis was performed on 30 $\mu$g of total RNA extracted from the buds of different organs at 11.5 and 13.5 dpc. Em, total embryo; UGR, urogenital ridges; Lu, lung; Li, liver; Lb, limb buds; T, testis; M, mesonephric tissue. X-ray films were exposed for 14 days with intensifying screens. (Bottom) Densitometric scanning of the autoradiograms. The results of one representative experiment of two performed are reported.

### *Dissociation Experiments*

Testes were isolated from 13.5 dpc embryos and disaggregated by incubation in trypsin–EDTA according to Pesce *et al.* (1994). The resulting cell suspension was seeded in a 12-well tissue culture dishes (50,000 cells/well) in DMEM alone, supplemented with HGF (150 U/ml), or with 10% FCS. After 4 h of culture, tritiated thymidine (1 $\mu$Ci/ml; New England Nuclear Corp., Boston, MA; sp act 20 mCi/mmol) was added and the culture was continued for an additional 16 h. At the end of the incubation time, cells were extensively washed with PBS and the amount of labeled thymidine incorporated into the cells was evaluated by TCA precipitation.

## RESULTS

### *Expression of HGF Receptor (c-met) mRNA in Developing Gonads*

***Northern blot analysis.*** We studied the temporal expression pattern of c-met during gonadal development. Intact UGR (containing both mesonephros and gonadal primordium) from 11.5 dpc mouse embryos have been explanted. Total RNA was extracted from male UGR as well as from limb buds, liver, lung, and from total embryo. Testes from 13.5 dpc embryos were also isolated and total RNA was extracted as well as from limb bud, liver, lung, and mesonephros from 13.5 dpc embryos. The presence of one c-met-specific transcript was detected by Northern blot in the RNAs extracted from UGR (11.5 dpc) and from embryonic testes (13.5 dpc), as shown in Fig. 1(top). The molecular weight of the detected mRNA species is estimated 9 kb since it is coincident with the single mRNA species detectable in the postnatal liver RNA. As expected, c-met expression was detected in the other embryonic organs and the expression levels were different between 11.5 and 13.5 dpc, increasing with age in developing lung and decreasing in liver and limb bud as indicated by the densitometric scanning of the bands (Fig. 1, bottom). C-met expression in testes of the different ages cannot be compared because 11.5 dpc testes have been isolated together with mesonephroi.

***Hybridization experiments.*** Male UGR were isolated from embryos at 11.5, 12.5, and 13.5 dpc and c-met expression was localized in the developing genital ridges by whole mount *in situ* hybridization experiments. At 11.5 dpc c-met expression was undetectable in the genital ridge (Fig. 2A, arrow) whereas detectable expression levels were found in the mesonephric tissue and high level in the pronephric tissue (Fig. 2A). At 12.5 dpc c-met expression was clearly detectable in the testicular cords (Fig. 2C, arrow) and a higher expression was detected at 13.5 dpc in the cords (Fig.

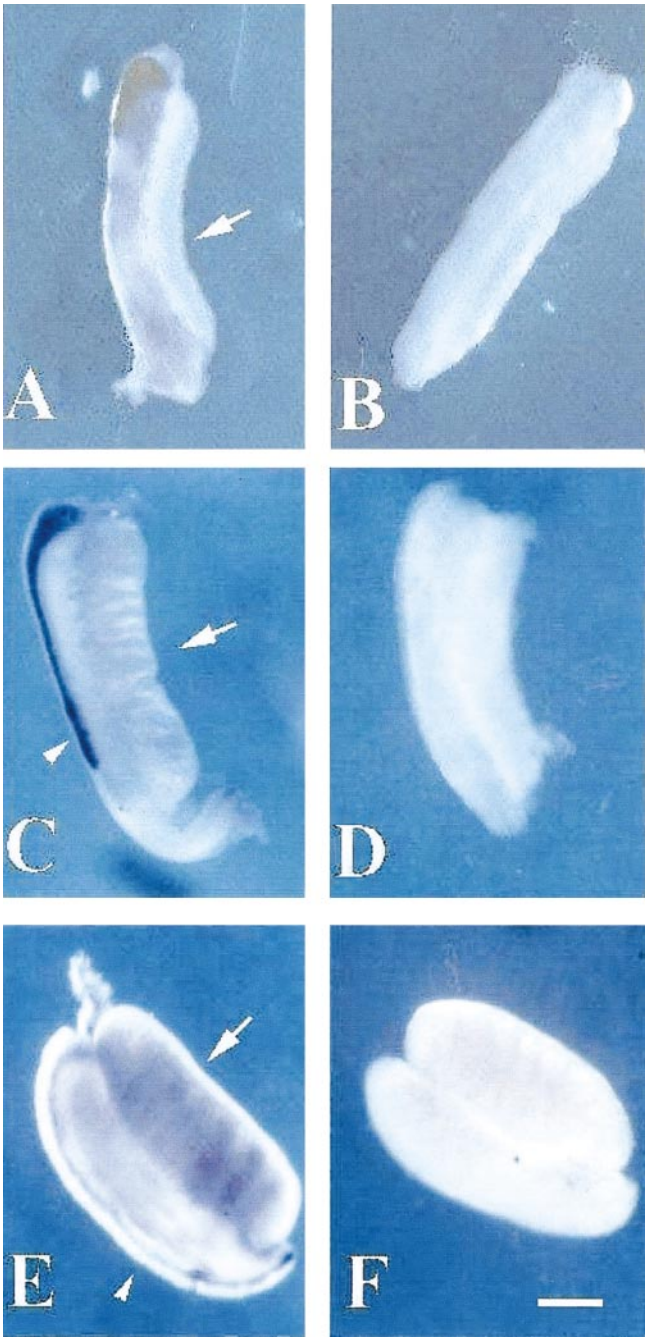


**FIG. 2.** Whole mount *in situ* hybridization of c-met expression during testis development. Arrows indicate developing male gonads at 11.5 dpc (A), 12.5 dpc (C), and 13.5 dpc (E). The hybridization with the sense probe at the same ages (B, D, and F, respectively) are reported. The arrowheads in C and E indicate the Mullerian duct. Bar, 180 $\mu$m.

2E, arrow). Mullerian duct showed a strong c-met expression at 12.5 dpc (Fig. 2C, arrowhead) whereas c-met expression was reduced at 13.5 dpc (Fig. 2E, arrowhead). Control samples obtained utilizing a sense probe (Figs. 2B, 2D, and 2F) did not give detectable signals. Hybridized samples were embedded in gelatin and sectioned to look at the internal morphology. Figure 3A shows a section of a 13.5 dpc testis. The peripheral part of the section (TC), that is, one longitudinally sectioned testicular cord, appears clearly labeled whereas the internal portion of the section, representing the precursor of the interstitial tissue, is not labeled in a detectable way. A transverse section of the 13.5 dpc mesonephros including the section of the genital ducts is also shown (Fig. 3B) indicating that Mullerian duct (M) expresses HGF receptor whereas Wolffian duct (W) is negative.

### *Role of HGF in Developing Testis*

***HGF stimulates testicular cord formation.*** To asses the biological function of HGF in testis development, UGR

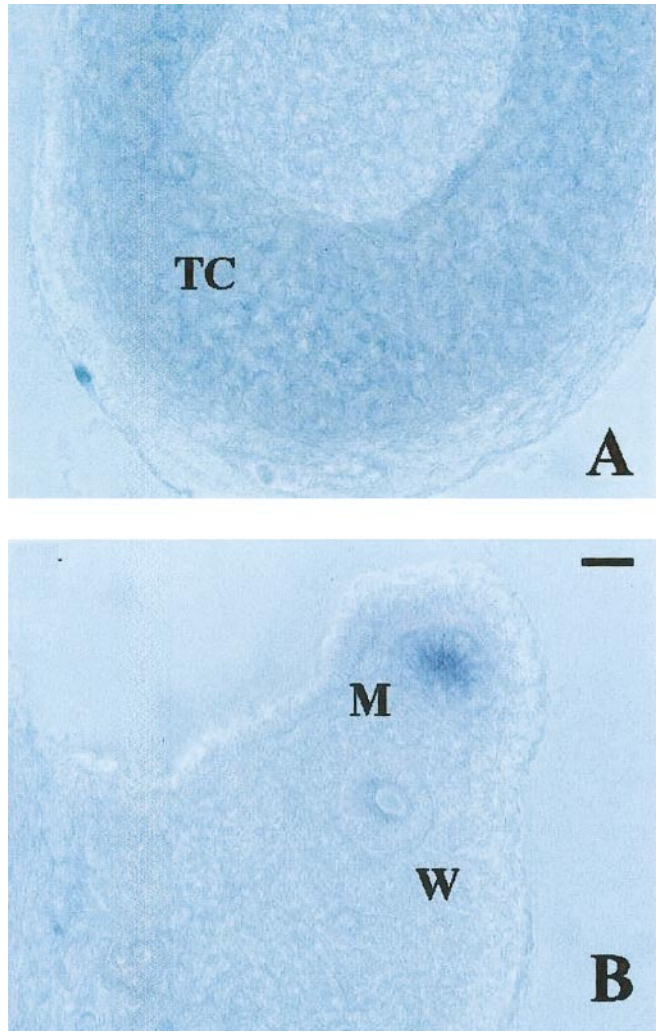


**FIG. 3.** (A) Section of a 13.5 dpc male gonad after *in situ* hybridization (bright field). The section shows a c-met-positive, longitudinally sectioned, testicular cord (TC). (B) Section containing the genital ducts is shown. Mullerian duct (M) is clearly positive, whereas Wolffian duct (W) appears negative. Bar, 26 $\mu$m.

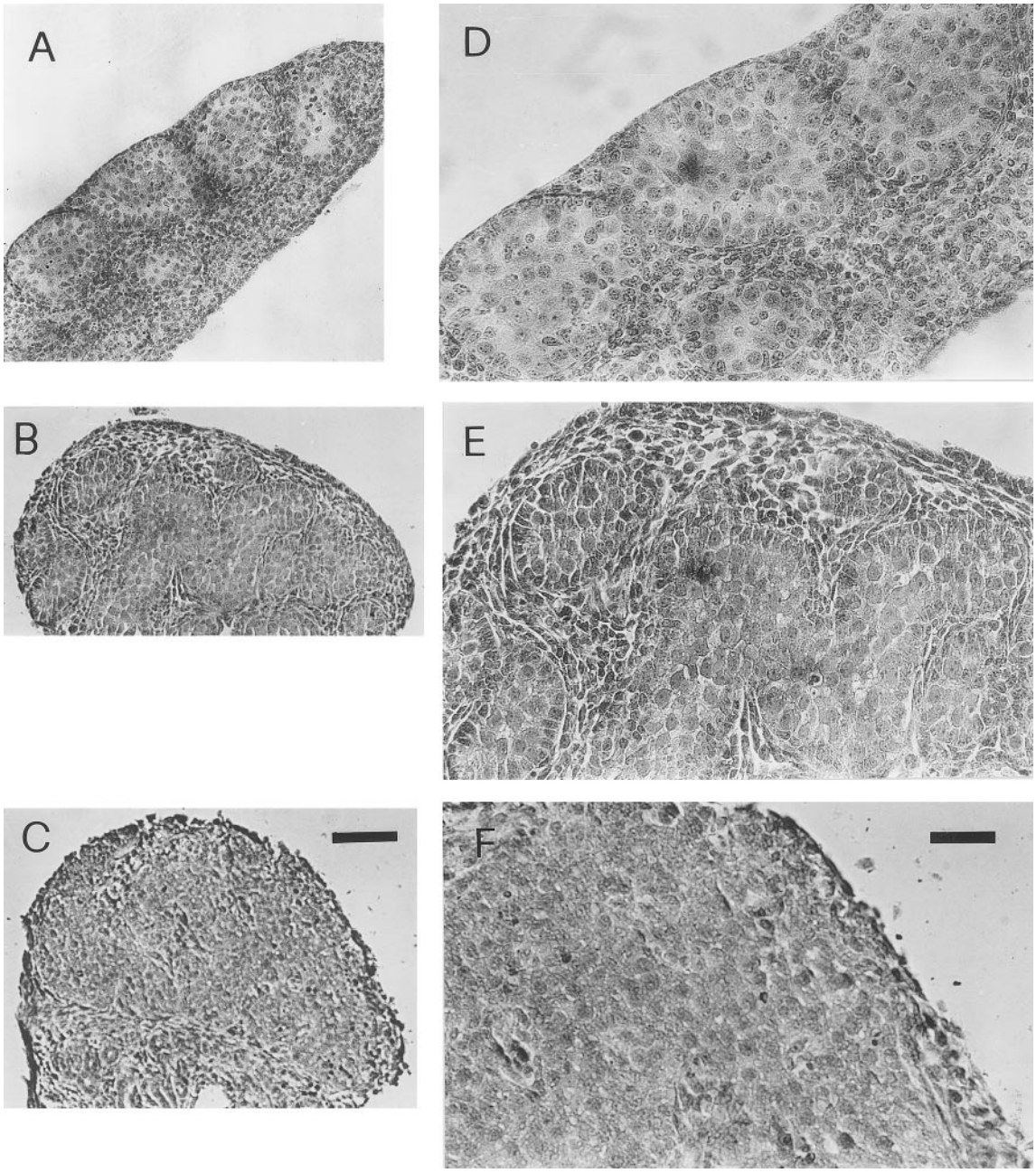


**FIG. 4.** Light microscopy of male 11.5 dpc UGR cultured in the presence of serum (A, D) or HGF (B, E) or medium alone (C, F). Low (A–C; bar, 76 $\mu$m) and high magnifications (D–F; bar, 38 $\mu$m) are presented.

isolated from 11.5 dpc embryos were cultured in presence of DMEM alone, DMEM supplemented with 10% FCS or supplemented with HGF (200–400 U/ml). Urogenital ridges cultured in medium supplemented with 10% FCS were able to differentiate and testicular cord formation was obtained after 4 days of culture (Figs. 4A and 4D) as previously described (Merchant-Larios *et al.*, 1993; Buehr *et al.*, 1993; Moreno-Mendoza *et al.*, 1995). By culturing the UGR in medium without serum but supplemented with HGF, testicular cord formation was obtained (Figs. 4B and 4E). Doses of HGF ranging between 200 and 400 U/ml were able to induce testis differentiation. At the end of the culture time, the morphology of the cords present in the HGF-treated UGR was not distinguishable from the morphology of the cords obtained culturing UGR in the presence of serum. Under both cultural conditions the formation of solid cordonal structures was clearly observable (Figs. 4A, 4D, 4B, and 4E). When culturing UGR in medium without any

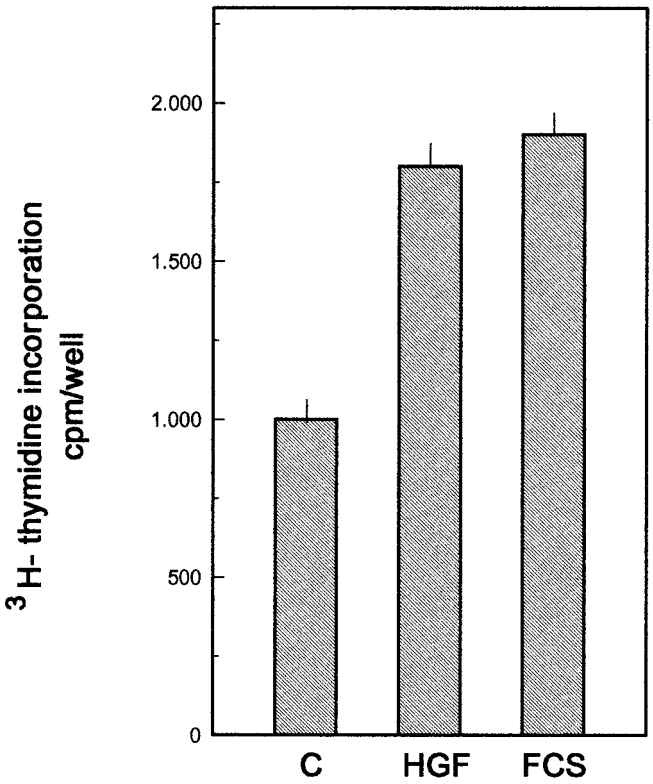


**FIG. 5.** Thymidine incorporation in cells isolated from 13.5 dpc testis and cultured for 24 h in the presence of hepatocyte growth factor (HGF) or 10% fetal calf serum (FCS) or in control medium (C). The tritiated precursor was supplemented for the last 16 h of culture. The results (means ± SD) of one representative experiment performed in duplicate are reported.

supplementation, the gonadal ridges survive during culture; however, they did not differentiate, at least at morphological level (Figs. 4C and 4F). Similar experiments were performed utilizing different growth factors such as TGF$\beta$, bFGF, PDGF-BB, and FSH. The reported substances did not support testicular cord formation, with the exception of PDGF which induced a morphological differentiation similar to the HGF-treated UGR (data not shown).

***HGF mitogenic effect.*** Testes isolated from 13.5 dpc embryos were disagreggated by incubation in trypsin as previously described by Pesce *et al.* (1994). The cell suspension obtained was cultured in the presence of medium alone, supplemented with HGF (150 U/ml) or with 10% FCS. Cells were labeled with tritiated thymidine for 16 h at the end of the first day of culture. The amount of labeled precursor incorporated into the cells was evaluated and the results showed that the cells cultured in the presence of HGF or serum were significantly more active in thymidine incorporation respect to the nontreated cells (Fig. 5).

To evaluate if HGF mitogenic effect was exerted on a specific cell type of the developing gonad, organ cultures of 13.5 dpc testes were performed in medium alone, in the presence of 10% serum or HGF. Bromodeoxyuridine was added to label the dividing cells and then samples were processed as indicated under Materials and Methods and studied at the optical microscope to detect BrdU-positive cells. In the different samples we did not find significant differences in the ratio of the labeled cells localized either inside or around the cords. The regions of the testes not yet morphologically differentiated were heavily labeled in both samples and cell count was impossible to perform (data not shown).

## DISCUSSION

In this study we report the expression of the gene encoding the HGF receptor, c-met, during mouse testis development. Moreover, the putative role of HGF in testicular cord formation has been investigated and its mitogenic effect on gonadal cells was evaluated. In the literature no data are available on these subjects and we first report that c-met is expressed in the embryonic male gonads at 12.5 dpc, that is, when morphological differentiation of the testis starts and testicular cords begin to be detectable. At 11.5 dpc, when the gonads are clearly distinguishable from the adjacent mesonephros, although morphologically indifferent, c-met is expressed in the urogenital complex (UGR), only localized to the mesonephric tissue, as indicated by the reported *in situ* experiments. The expression of c-met in other differentiating organs has been previously reported. C-met expression has been detected during the entire development of lung, pancreas, and kidney and transiently expressed in the developing muscles and in the ventral horns of the spinal cord during motoneurons development (Sonnenberg *et al.*, 1993). Successive studies have pointed out that c-met, transducing HGF multiple biological effects, has an important role in the metanephros development, in particular, in the branching of the ureteric bud and in the differentiation of the metanephric mesenchymal cells into nephrons (Santos *et al.*, 1994; Woolf *et al.*, 1995). Moreover, the activation of c-met induces branching morphogenesis of different mammary epithelial cell lines in culture (Brinkmann *et al.*, 1995; Soriano *et al.*, 1995) and during lung development which can be inhibited by anti-HGF antibody (Ohmichi *et al.*, 1998). All these reports indicate that, besides its mitogenic and motogenic activity, this growth factor is involved in the formation of the tubular-like structures during embryonic differentiation.

In line with the reported effect of HGF in the development of different organs, we describe in this paper the ability of HGF in supporting the *in vitro* differentiation of the mouse male gonad. It is known that urogenital ridges isolated from 11.5 dpc male embryos can be maintained *in vitro*. During culture, testicular cord formation occurs and well-organized structures are detectable after 4 days of culture in the presence of serum (Merchant-Larios *et al.*, 1993; Buehr *et al.*, 1993; Moreno-Mendoza *et al.*, 1995). Serum addition is essential for testis differentiation since, as we observed, cord formation was not observable at light

microscopy when culture was performed in the absence of serum. During culture, cell migration from the mesonephros to the male gonad occurs, induced by one or more proteic, uncharacterized substance(s) produced by the male gonad which triggers cell migration. When culturing gonads from wild-type mice and mesonephroi from a transgenic strain expressing $\beta$-galactosidase, it has been demonstrated that cells derived from the mesonephroi contribute to the testicular endothelial cell population, to the myoepithelial cells surrounding the vessels and to the peritubular myoid cell population localized around testicular cords (Martineau *et al.*, 1997). We have cultured UGR in the presence of HGF, without any serum addition, and we have observed a testicular differentiation morphologically identical to the differentiation obtained culturing UGR in the presence of serum. We demonstrate that c-met is expressed and functionally active during testis morphogenesis and that HGF addition to the undifferentiated 11.5 dpc male gonads is sufficient to induce testicular cord formation. Therefore, our results indicate that HGF is able to induce the cellular events necessary for the differentiation of the "indifferent" male gonad. We have detected c-met expression in the developing testis starting from 12.5 dpc; however, HGF exerts its morphogenetic role on the UGR at 11.5 dpc. We can hypothesize that c-met is already expressed in 11.5 dpc testis at very low levels, undetectable by the technique we used. Conversely, it is conceivable that HGF affects the mesonephric tissue that, as we detected, expresses c-met at 11.5 dpc. In the latter case gonadal differentiation could be due to factors produced by the mesonephric tissue under HGF stimulation which, in turn, stimulate testis morphogenesis. Alternatively, gonadal differentiation could be due to the HGF-induced cell migration from the mesonephros to the differentiating gonad. Other growth factors involved in testicular development, such as TGF$\beta$ and bFGF (Koike and Noumura, 1994; Cancilla and Risbridger, 1998; Cupp *et al.*, 1999) and FSH, regulating fetal Sertoli cell metabolic activities (Rouiller-Fabre *et al.*, 1998), did not sustain testicular differentiation. Conversely, PDGF induced the formation of morphologically defined cords. This finding is in line with the knowledge that PDGF influences lung branching morphogenesis (Souza *et al.*, 1995) and that all three of the PDGF isoforms act as chemoattractants for peritubular myoid cells, with PDGF-BB being the most potent attractant (Gnessi *et al.*, 1995).

In conclusion, the detected expression of c-met in the beginning period of testis differentiation and the detected ability of HGF in inducing testicular differentiation suggest that this growth factor, besides its role as mitogenic factor, plays a fundamental role in cell migration and/or cell differentiation during testicular cord formation.

## ACKNOWLEDGMENTS

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